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(54) Title: USE OF GINKGO EXTRACT

(57) Abstract: The present invention is directed to the use of the extract of Ginkgo biloba leaves or isolated Ginkgolide B (GKB), a component of the extract of Ginkgo biloba leaves in a method for decreasing the expression of peripheral-type benzodiazepine receptor (PBR) in cells of a patient in need thereof. Further, the present invention is directed to the use of the extract of Ginkgo biloba leaves or isolated GKB in a method for decreasing the proliferation of cancer cells in a patient. More particularly, the present invention is directed to the use of the extract of Ginkgo biloba leaves or isolated GKB in a method of decreasing cancer cell proliferation in a patient wherein the cancer cell is human breast cancer cell. Even more particularly, the present invention is directed to the use of the extract of Ginkgo biloba leaves or isolated GKB in method of decreasing cancer cell proliferation in a patient wherein the cancer cell is of the aggressive and invasive phenotype and expresses high levels of PBR in comparison to non-aggressive cancer cell.

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the terpene constituents of EGB 761®, ginkgolides and bilobalide, have anti-oxidant properties (Pietri, S., Maurelli, E., Drieu, K., and Culcasi, M., J. Mol. Cell. Cardiol., 29: 733-742, 1997; Yao, Z., Boujrad, N., Drieu, K., and Papadopoulos, V., Adv. Ginkgo Biloba Res. 7: 129-138, 1998). Other studies of EGB 761® have reported medicinal value of the product

5 in the treatment of a variety of clinical disorders including cerebrovascular and peripheral vascular insufficiencies associated with aging and senility. See e.g., Ginkgo biloba Extract (EGB 761®) Pharmacological Activities and Clinical Applications, DeFeudis, F.V., Eds, Elsevier, 1991; and Ullstein Medical 1998, Ginkgo biloba extract (EGB 761®), Eds. Wiesbaden, DeFeudis, F.V. The extract contains 24% ginkgo-flavone glycosides, 6%

10 terpene lactones (ginkgolides and bilobalide), about 7% proanthocyanidins and several other constituents. See Boralle, N., et al., In: Ginkgolides, Chemistry, Biology, Pharmacology and Clinical perspectives, Ed: Braquet, P., J.R. Prous Science Publishers, 1988.

Tumor progression is a multi-step process in which normal cells gradually acquire

15 more malignant phenotypes, including the ability to invade tissues and form metastases, the primary cause of mortality in breast cancer. During this process, the "aberrant" expression of a number of gene products may be the cause or the result of tumorigenesis. Considering that the first step of tumor progression is cell proliferation, it can be proposed that tumorigenesis and malignancy are related to the proliferative potential of tumoral cells.

20 Studies in a number of tumors such as rat brain containing glioma tumors (Richfield, E.K. *et al.* (1988) *Neurology* 38:1255-1262), colonic adenocarcinoma and ovarian carcinoma (Katz, Y. *et al.* (1988) *Eur. J. Pharmacol.* 148:483-484 and Katz, Y. *et al.* (1990) *Clinical Sci.* 78:155-158) have shown an abundance of peripheral-type benzodiazepine receptors (PBR) compared to normal tissue. Moreover, a 12-fold increase in PBR density relative to

25 normal parenchyma, was found in human brain glioma or astrocytoma (Comu, P. *et al.* (1992) *Acta Neurochir.* 199:146-152). The authors suggested that PBR densities may reflect the proliferative activity of the receptor in these tissues. Recently, the involvement of PBR in cell proliferation was further shown (Neary, J.T. *et al.* (1995) *Brain Research* 675:27-30; Miettinen, H. *et al.* (1995) *Cancer Research* 55:2691-2695), and its expression of

30 human astrocytic tumors was found to be associated with tumor malignancy and proliferative index (Miettinen, H. *et al. supra*; Alho, H. (1994) *Cell Growth Different.* 5:1005-1014). Further studies have shown that PBR receptors are abundant in human

cells, indicating that the cholesterol transport mechanism was impaired. Further cholesterol transport experiments in bacteria expressing the 18-kDa PBR protein provided definitive evidence for a function as a cholesterol channel/transporter (Li and Papadopoulos, V. *et al.* (1998) *Endocrinology*).

5 We hypothesized that the peripheral-type benzodiazepine receptor is part of the changes in cellular and molecular functions that account for the increased aggressive behavior in cancer, and we chose to examine this hypothesis in human breast cancer. Breast cancer is the most common neoplasm and the leading cause of cancer-related deaths for women in most developing countries (Lippman, M. E. (1993) *Science* 259:631-  
10 632), affecting nearly 184,000 women, with over 46,000 deaths annually in the U.S. alone (American Cancer Society, 1996). Human breast cells are unlike brain and gonadal cells and cannot produce steroids, but like many other cells in the body, are able to metabolize steroids.

15 Increased PBR expression correlates with increased aggressive behavior of tumor cells. Invasive tumors invade and grow locally but they do not metastasize. However, the aggressive tumors have the ability to invade and metastasize through the blood vessels to different places of the human body. Tumor metastasis into vital organs (such as lungs) is the most common cause of death.

20 The correlation between high levels of expression of PBR and metastatic potential in for human breast cancer is shown in copending U.S. Application No. 09/047,652 filed March 25, 1998, in which Vassilios Papadopoulos of the instant application is a co-inventor. However, due to the involvement of PBR in cell proliferation, and the expression of PBR in all cells, it is likely that this correlation would exist for other solid tumors and cancers such as prostate cancer, colon cancer, brain tumors, and tumors in steroid producing tissues  
25 such as gonadal tumors, to name a few.

#### **Summary of the Invention**

In one aspect, the present invention is directed to a method of combating cancer in a patient in need of such combating, wherein the cancer is caused by the deregulation of expression of proteins having a role in regulating tumor cells, which comprises administering  
30 an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

In another aspect, the present invention is directed to a method of combating the proliferation of cancer cells in a patient in need of such combating, wherein the proliferation is caused by the deregulation of expression of proteins having a role in regulating tumor

administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

1 In another aspect, the present invention is directed to a method of decreasing the expression of cell cycle regulators prothymosin- $\alpha$ , CDK2, p55CDC, myeloblastin and p120  
5 proliferating-cell nuclear antigen (PCNA) in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

10 In another aspect, the present invention is directed to a method of decreasing the expression of intracellular signal transduction modulators NET1 and ERK2, in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

15 In another aspect, the present invention is directed to a method of decreasing the expression of apoptosis-related proteins Adenosine A2A Receptor, Flt3 ligand, Grb2, Clusterin, RXR- $\beta$ , Glutathione S-transferase P, N-Myc, TRADD, SGP-2 and NIP-1, in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

20 In another aspect, the present invention is directed to a method of decreasing the expression of transcription factors Id-2, ATF-4, ETR101 and ETR-103 in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

25 In another aspect, the present invention is directed to a method of decreasing the expression of growth factors macrophage colony-stimulating factor-1, heparin-binding EGF-like growth factor, hepatocyte growth factor-like protein and inhibin  $\alpha$ , in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

30 In another aspect, the present invention is directed to a method of decreasing the expression of cell adhesion molecules CD19 B-lymphocyte antigen, L1CAM,  $\beta$ -catenin, integrin subunits  $\alpha$ 3,  $\alpha$ 4,  $\alpha$ 6,  $\beta$ 5, and  $\alpha$ M, in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.

In another aspect, the present invention is directed to a method of decreasing the expression of genes APC, PE-1, RhoA, c-Jun, prothymosin- $\alpha$ , CDK2, p55CDC, myeloblastin, p120 proliferating-cell nuclear antigen (PCNA), NET1, ERK2, Adenosine A2A

autoradiogram of the blot. PBR migrates at 0.9Kb. Bottom, relative intensity of the PBR mRNA/28S ribosomal RNA visualized by ethidium bromide staining. The autoradiogram and PBR mRNA quantitation represent one out of two independent experiments.

**Figure 4.** Effect of EGB 761® on MDA-231 cell proliferation. MDA-231 cells grown in 96-well plates were washed with PBS and cultured in media supplemented with 10% FBS in the presence or absence of the indicated concentrations of EGB 761®. 4h prior to the end of incubation, bromodeoxyuridine (BrdU) was added to each well. Incorporation of BrdU was measured at 450nm (reference=700nm). Data points represent the mean  $\pm$  S.D. of four independent experiments carried out in quadruplicate. One-way ANOVA indicates that MDA-231 cell proliferation was significantly altered by treatment with EGB 761® at 48, 72 and 96 h timepoints ( $P<0.0001$ ).

**Figure 5 Right, Middle, Left.** Recovery of MDA-231 cell proliferation upon removal of EGB 761®. MDA-231 cells grown in 96-well plates were washed with PBS and cultured in media supplemented with 10% FBS in the presence or absence of 2 (Left), 20 (Middle) or 200 (Right)  $\mu\text{g/ml}$  EGB 761® for 48 h. At the end of the treatment the cells were washed and incubated in EGB 761®-free media for 48 h. 4h prior to the end of incubation, bromodeoxyuridine (BrdU) was added to each well. Incorporation of BrdU was measured at 450nm (reference=700nm). Data points represent the mean  $\pm$  S.D. of two independent experiments carried out in quadruplicate.

**Figure 6.** Effect of GKB on MDA-231 cell proliferation. MDA-231 cells grown in 96-well plates were washed with PBS and cultured in media supplemented with 10% FBS in the presence or absence of either 2  $\mu\text{g/ml}$  or 20  $\mu\text{g/ml}$  GKB for 48 hours. 4h prior to the end of incubation, bromodeoxyuridine (BrdU) was added to each well. Incorporation of BrdU was measured at 450nm (reference=700nm). Data points represent the mean  $\pm$  S.D. of two independent experiments carried out in quadruplicate. One-way ANOVA indicates that MDA-231 cell proliferation was significantly altered by treatment with GKB ( $P<0.0001$ ).

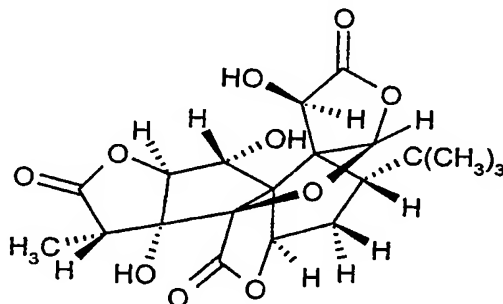
**Figure 7.** Effect of EGB 761® and GKB on MCF-7 cell proliferation. MCF-7 cells were grown in 96-well plates as described in Figure 4 for the MDA-231 cells. MCF-7 cells were treated for 48 hours without or with either 20 (EGB-20) or 200 (EGB-200)  $\mu\text{g/ml}$  EGB 761®, or 2 (GKB-2) or 20 (GKB-20)  $\mu\text{g/ml}$  GKB. 4h prior to the end of incubation, bromodeoxyuridine (BrdU) was added to each well. Incorporation of BrdU was measured at 450nm (reference=700nm). Data points represent the mean  $\pm$  S.D. of two independent experiments carried out in quadruplicate. One-way ANOVA indicates that MCF-7 cell

middle of the tumor obtained from animals treated with EGB 761®. F shows the PBR immunostaining in cells found in the middle of the tumor obtained from animals treated with GKB. G shows the PBR immunostaining in cells found in the edge of the tumor obtained from animals treated with EGB 761®. H shows the PBR immunostaining in cells found in the middle of the tumor obtained from animals treated with GKB. Arrowheads indicate nuclei. Magnification is x75 for A, B, C, E, F, G, and H and x150 for C.

#### Detailed Description

The term "ginkgo terpenoid" includes all of the naturally occurring terpenes which are derived from the gymnosperms tree *Ginkgo biloba* as well as synthetically produced ginkgo terpenoids and pharmaceutically active derivatives and salts thereof and mixtures thereof. Examples of ginkgo terpenoids include ginkgolides. Examples of ginkgo terpenoids are disclosed in *Ginkgolides, Chemistry, Biology, Pharmacology, and Clinical Perspectives*, J.R. Provs. Science Publishers, Edited by P. Braguet (1988); F.V. DeFeudis, *Ginkgo Biloba Extract (EGB 761®); Pharmacological Activities and Clinical Applications*, Elsevier, Chapter II (1991).

The term "ginkgolide" as used herein include the various ginkgolides disclosed in the books cited above as well as non-toxic pharmaceutically active derivatives thereof. Examples of ginkgolide derivatives include tetrahydro derivatives, acetyl derivatives, and alkyl esters such as the monoacetate derivatives and triacetate derivatives disclosed in Okabe, et al., *J. Chem. Soc. (c)*, pp. 2201-2206 (1967). Ginkgolide B has the following structure and as used herein, refers to isolated ginkgolide B:



The term "Ginkgo biloba extract" as used herein includes a collection of natural molecules, including terpenoids, derived from the leaves of the *Ginkgo biloba* tree. Preferably, the extract is the specific formulation of *Ginkgo biloba* extract known as EGB 761®.

invention include but are not limited to enzyme labels, radioisotopic labels, non-radioactive isotopic labels and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include  $^3\text{H}$ ,  $^{111}\text{In}$ ,  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{75}\text{Se}$ ,  $^{152}\text{Eu}$ ,  $^{90}\text{Y}$ ,  $^{67}\text{Cu}$ ,  $^{21}\text{Ci}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Pb}$ ,  $^{47}\text{Sc}$ ,  $^{109}\text{Pd}$ ,  $^{11}\text{C}$ ,  $^{19}\text{F}$ ,  $^{123}\text{I}$ , etc.

Examples of suitable non-radioactive isotopic labels include  $^{157}\text{Gd}$ ,  $^{55}\text{Mn}$ ,  $^{162}\text{Dy}$ ,  $^{52}\text{Tr}$ ,  $^{46}\text{Fe}$ , etc.

Examples of suitable fluorescent labels include a  $^{152}\text{Eu}$  label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, etc.

We examined herein the effect of Ginkgo biloba extracts, more specifically EGB 761® and GKB on PBR expression and cell proliferation, particularly in human breast cancer cells. We used the highly aggressive cell line MDA-231, which expresses over 60-fold higher levels of PBR ligand binding and mRNA relative to the non-aggressive cell line MCF-7. EGB 761® and GKB decreased in a time- and dose-dependent manner PBR expression and cell proliferation in MDA-231 cells whereas EGB 761® and GKB did not affect the MCF-7 cell proliferation to the same degree. This effect was reversible and it was not due to the antioxidant properties of the compounds tested.

The determination of elevated levels of PBR is done relative to a sample with no detectable tumor. This may be from the same patient or a different patient. For example, a first sample may be collected immediately following surgical removal of a solid tumor. Subsequent samples may be taken to monitor recurrence of tumor growth and/or tumor cell proliferation. Additionally, other standards may include cells of varying aggressive phenotype such that an increase or decrease in aggressive phenotype can be accessed.

The distinct sub-cellular localization of PBR in the cytoplasm of epithelial cells of normal breast ducts and the absence of staining in the nucleus and the perinuclear area of

**Protein Measurement.** Protein levels were measured by the Bradford method (Bradford, MM, 1976, Anal. Biochem., 72: 248-254) using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

**RNA (Northern) Analysis.** PBR mRNA expression in MDA-231 cells treated with the

5 various compounds was examined by Northern Blot analysis as we previously described (Hardwick, M., et al., 1999, Cancer Research, 59: 831-842). In brief, total cellular RNA was isolated using the RNazol B reagent (TEL-TEST, Inc., Friendswood, TX) and chloroform. 20µg of total RNA from each cell line were run on 1% agarose gels and transferred overnight to nylon membranes (S&S Nytran, Schleicher & Schuell, Keene, NH) (21). A 0.2  
10 kb human PBR (hPBR) cDNA fragment (derived from the pCMV5-PBR plasmid vector containing the full length hPBR kindly given by Dr. Jerome Strauss, University of Pennsylvania, PA) was radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a random primers DNA labeling system (Life Technologies, Gaithersburg, MD). The hybridization conditions were as we previously described (Hardwick, M., et al., 1999, Cancer Research, 59: 831-842).  
15 Autoradiography was performed by exposing the blots to X-OMAT AR film (Kodak, Rochester, NY) at -70°C for 4-48hr. Quantification of PBR mRNA was carried out using the SigmaGel software (Jandel Scientific, San Rafael, CA).

**Nucleic Acid Arrays.** We used the Atlas human cDNA expression array I from Clontech (Palo Alto, CA). This array contains 588 human PCR-amplified cDNA fragments of 200-500

20 bp long immobilized on a positively charged nylon membrane. MDA-231 cells were treated with and without 20 µg/ml EGB 761® for 48 hours. Poly A+ RNA was isolated from control and EGB 761®-treated cells. <sup>32</sup>P-labeled cDNA probes were generated from each poly A+RNA and hybridized to the Atlas array according to the manufacturer's recommendations.

Autoradiography was performed by exposing the blots to X-OMAT AR film (Kodak,  
25 Rochester, NY) at -70°C for 4-96 hr. Quantification of the hybridization seen was carried out using the SigmaGel software (Jandel Scientific, San Rafael, CA). Multiple exposures were

used in order to detect genes expressed at low levels. The three internal controls, ubiquitin, G3PDH and  $\beta$ -actin were used to compare the relative expression levels of the detected gene products in the control and EGB 761®-treated cells. Experimental variations were

30 corrected using the ratios of gene expression versus the internal controls. The effect of the EGB 761® treatment on each gene product is expressed as % of control (untreated) cells. The results presented herein show genes affected consistently, at a level above 30% of control, by the EGB 761® treatment.



for 1h. Secondary antibody reactions were performed using horseradish peroxidase-coupled goat anti-rabbit secondary antibody diluted 1:500 in PBS supplemented with 10% calf serum. After washing the slides three times in PBS for 2 min each, fresh H<sub>2</sub>O<sub>2</sub> diluted 1:1,000 with 3-amino-9-ethyl carbazole (AEC) was added and slides were incubated for 1h at 37°C. The slides were then rinsed in distilled H<sub>2</sub>O before mounting with Crystal/Mount.

**Statistical Analysis.** Comparison of multiple means was performed with InStat's one-way analysis of variance (ANOVA) (GraphPad Inc., San Diego, CA). All *F* statistics and *P* values for one-way ANOVAs are provided in the text. Comparison of individual drug treatments to the control treatments was performed with unpaired *t*-test. All *p* values for unpaired *t*-tests are provided in the text.

## RESULTS

**EGB 761® and GKB reduce the PBR Ligand Binding Capacity of the MDA-231 Human Breast Cancer Cells.** Figure 1 shows that increasing concentrations of the injectable form of EGB 761® inhibit in time-dependent manner the PBR ligand binding capacity (B<sub>max</sub>), determined using saturation isotherms with the radiolabeled ligand PK 11195 followed by Scatchard analysis of the data. Similar results were obtained using isolated GKB (Figure 2). Interestingly, EGB 761® and GKB decreased PBR levels by 66% of control values. No significant effects on the receptor affinity (K<sub>d</sub>) could be seen ( $5.8 \pm 1.4$  pmol/mg protein, n=12).

**EGB 761® and GKB reduce the PBR mRNA Expression in MDA-231 Human Breast Cancer Cells.** RNA (Northern) blot analysis was performed in order to determine if the differences seen in PBR ligand binding between the control and the EGB 761®- or GKB-treated cells reflect an effect on PBR mRNA expression. As shown in Fig. 3, both EGB 761® and GKB reduced PBR mRNA levels. This result fits with the results presented above on the PBR ligand binding expression.

**EGB 761® and GKB Inhibit MDA-231 Cell Proliferation.** Using the Bromodeoxyuridine (BrdU) Cell Proliferation ELISA (Boehringer-Mannheim, Indianapolis, IN), we examined the effect of increasing concentrations of EGB 761® on MDA-231 cell proliferation. Fig. 4 shows that EGB-761 inhibits in a concentration- and time-dependent manner the MDA-231 cell proliferation. This effect of EGB 761® was reversible, even for the highest concentration of EGB 761® used. (Fig. 5). Incubation of MDA-231 cells for 48 hours with EGB 761®, followed by washing and incubation for another 48 hours in EGB 761®-free medium,

Mamm. Gland. Biol. Neopl. 1:5-19, 1996). Fig. 10 shows that 30 days treatment with either 50 mg/kg EGB 761® or with 1 mg/kg GKB resulted in a 35% ( $p=0.037$ ) and 32% ( $p=0.043$ ) decrease in tumor size, measured a month after the end of the treatment, respectively. These treatments did not affect the animal body weight (data not shown). Considering these

5 *in vitro* data on the effect of EGB 761® and GKB on PBR expression in MDA-231 cells, we examined whether EGB 761® and GKB also decreased PBR expression in the MDA-231 xenografts. Fig. 11(A-D) shows horseradish peroxidase (HRP) staining of the PBR antiserum used to detect the 18,000 molecular weight protein in MDA-231 xenografts from vehicle-treated animals. The hematoxylin counterstain was omitted in order to distinguish

10 the nuclear localization of PBR (19) in the tumors. Fig. 11A shows the middle of a tumor where the nuclear localization of the 18,000 PBR protein can be easily seen (see arrowheads). Fig 11B shows the immunostaining seen in the edge of the tumor obtained from vehicle-treated animals. A higher magnification of the immunostaining seen in the edge of the tumor obtained from vehicle-treated animals is shown in Fig. 11C, and a control treated

15 with a non-specific antiserum is shown in Fig. 11D. Treatment with either EGB 761® (Fig. 11E) or GKB (Fig. 11F) reduced the nuclear PBR expression present in cells found in the middle of the tumor. Interestingly, treatment with either EGB 761® (Fig. 11G) or GKB (Fig. 11H) also eliminated the nuclear PBR expression present in the cells at the edge of the tumors. However, in the later case cytosolic immunostaining could be seen (Figs. 11G and

20 H). These data were replicated in sections taken from xenografts grown in three separate animals.

It is of interest to note that even in the presence of high concentrations of either EGB 761® or GKB, PBR levels and rates of cell proliferation could not be reduced below 30% of normal values. This suggests that there is a minimum of PBR required to maintain

25 membrane integrity and cell function. It should be also noted that even at the highest concentrations used, neither EGB 761® nor GKB were toxic for the cells, because cell proliferation recovered upon removal of the compounds. These data suggest that these compounds are cytostatic and not cytotoxic. Additional cytotoxicity assays indicated that under the same conditions neither EGB 761® nor GKB induced any significant cell death.

30 The absence of any significant decrease in the amount of reactive oxygen species produced in the MDA-231 cells by EGB 761® or GKB suggests that their anti-oxidant properties were not responsible for decreasing PBR expression and cell proliferation in the

tested were effective only on the MDA-231-cells, which express high levels of PBR, these data suggest that the expression of nuclear PBR may be a determining factor for a tumor cell to acquire an aggressive and invasive phenotype.

5

**Table I:** Effect of EGB 761® on MDA-231 gene expression examined using the Atlas human cDNA expression array as described under Materials and Methods.

<u>Name</u>	<u>% Change</u>	<u>Function</u>	<u>References</u>
<b><u>Oncogenes and Tumor Suppressors</u></b>			
c-Myc	+75%	-basic helix-loop-helix-leucine zipper transcription factor -Myc/Max heterodimers induce cell-cycle progression, apoptosis, and malignant transformation	(37)
c-Jun	-78%	-part of the AP-1 transcription factor that regulates genes involved in cell proliferation	(38)
RhoA	-93%	-GTP-binding protein that is an important regulator of cell proliferation -RhoA inactivation inhibits HL60 cell proliferation	(39) (40)
APC	-59%	-APC mutations are associated with both hereditary and sporadic colorectal cancers	(41) (42)
PE-1	-42%	-a negative post-translational regulator of $\beta$ -catenin -transcription factor	(43)
<b><u>Cell Cycle Control Proteins</u></b>			
Prothymosin- $\alpha$	-79%	-acidic nuclear protein that is upregulated in proliferating thymocytes, lymphocytes from leukemia patients, and in malignant breast lesions	(44)
Myeloblastin	-66%	-a serine protease involved in leukemia cell differentiation	(45)
p55CDC	-63%	-similar to mitosis regulators CDC4 and CDC20	(46)
p120 Proliferating-cell Nuclear Antigen	-68%	-expression positively correlated with cell proliferation status -nucleolar protein expressed in proliferating cells	(47) (48)
CDK2	-83%	-adenocarcinoma -cyclin-dependent tyrosine kinase involved in progression through the cell cycle -cyclin E/Cdk2 inactivates the retinoblastoma tumor suppressor to allow the cell to progress to S phase -Vitamin D inhibition of LNCaP cell proliferation coincided with a reduction in Cdk2 activity	(49) (50)
<b><u>Intracellular Transducers</u></b>			
NET1	-55%	-RhoA-specific guanine exchange factor -NIH3T3-transforming protein	(51)
ERK2	-46%	-member of the extracellular signal-related protein kinase family -activated upon cell stimulation	(52)
<b><u>Apoptosis-Related Proteins</u></b>			
Adenosine A2A Receptor	-40%	-G protein-coupled receptor involved in the cAMP signaling pathway	(53)
Flt3 ligand	-58%	-ligand for the Flt3 cytokine receptor tyrosine kinase -induces proliferation of leukemic myeloid cells	(54)
Grb2	-70%	-an adapter protein that links receptor tyrosine kinases to the Ras/MAPK signaling pathway via its SH2 domain	(55)
Clusterin	-54%	-a glycoprotein associated with cell adhesion and apoptosis -increased expression is linked to Alzheimer's disease	(56, 57) (58)
RXR- $\beta$	-55%	-retinoid-activated transcription factor -inhibition of chondrocyte proliferation by retinoic acid causes a reduction in RXR- $\beta$ mRNA expression	(59) (60)
Glutathione S-transferase P	-39%	-a multi-drug resistance gene that is overexpressed in various human tumors	(61, 62) (63)

1999; Dang, C.V., Mol. Cel. Biol., 19: 1-11, 1999) and are strongly correlated with tumor cell proliferation. Previous studies have shown that arrest of neuroblastoma cell growth by the tyrosine kinase inhibitor genistein is accompanied by down-regulation of n-Myc expression. This data fits extremely well with our cell proliferation and n-Myc data. Overexpression of c-Myc, however, is associated with a stimulation of cell proliferation in normal serum conditions. Overexpression of c-Myc induces cell death in the absence of serum or other survival factors. Taken together the data implies that deregulation of c-Myc expression requires the altered expression of other genes, as well.

In our microarray experiment, we discovered the deregulated expression of several other c-Myc-related genes. One such gene, *prothymosin  $\alpha$*  (*proT  $\alpha$* ), is induced by c-Myc. However, expression of *proT  $\alpha$*  is reduced by 79% rather than increased, as might be predicted by up-regulation of c-Myc. Further, expression of other c-Myc target genes such as *cdc25A*, *cyclin A*, and *cyclin E* are unaffected by treatment of MDA-231 cells with EGB 761®, suggesting either a treatment-specific or a cell line-specific short circuit in c-Myc-regulated gene transcription. Other data gathered from the microarray experiment further supports this hypothesis. c-Myc transcriptional regulation is under the control of APC and  $\beta$ -catenin. However, both of these genes are down-regulated by EGB 761® in MDA-231 cells (59 and 58%, respectively) while c-Myc is up-regulated. While some of this data appears to be contradictory, much of the published data on the role of c-Myc in cell proliferation, differentiation, and cell death also appears contradictory.

Similar to the altered regulation of c-Myc and Myc-related proteins by EGB 761®, the microarray experiment exposed disruption of several signaling molecules. EGB 761® treatment resulted in a 93% reduction in the expression of *RhoA*, a gene encoding a GTP-binding protein involved in numerous cellular phenomena, and a 55% reduction in *NET1* expression, a RhoA-specific guanine exchange factor. Interestingly, *RhoA* has been demonstrated to regulate cyclin E/Cdk2 activity in fibroblasts. Activation of cyclin E/Cdk2 complex is crucial to the progression of the cell cycle from G1 to S-phase. Regulation of cyclin E/Cdk2 activity has also been demonstrated by c-Myc. Although the significance of these two phenomena is not immediately obvious, it should be noted that expression of *Cdk2* is reduced by 83% by EGB 761®.

Other important signaling molecules are also down-regulated by EGB 761®. Expression of the adapter molecule Grb2 is reduced by 70%. Grb2 plays an important role in cellular signaling by physically linking signal transducers such as receptor tyrosine

derivatives are optionally combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (16<sup>TH</sup> ED., Osol, A. ed., Mack Easton PA. (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly (methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

EGB 761® and isolated GKB can be administered by oral, parenteral (e.g., intramuscular, intraperitoneal, intravenous or subcutaneous injection, or implant), nasal, vaginal, rectal, sublingual or topical routes of administration and can be formulated with pharmaceutically acceptable carriers to provide dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than such inert diluents, e.g., lubricating agents such as magnesium stearate. In the case of capsules,

38. Huang, W. and Erikson, R.L. Signal Transduction. p. 161. Chapman and Hall, 1996.
39. Hu, W., Bellone, C.J., and Baldassare, J.J. RhoA stimulates p27(Kip) degradation through its regulation of cyclin E/CDK2 activity. *J. Biol. Chem.*, 274: 3396-3401, 1999.
- 5 40. Aepfelbacher, M., Essler, M., Lubner, D.Q., and Weber, P.C. ADP-ribosylation of the GTP-binding protein RhoA blocks cytoplasmic division in human myelomonocytic cells. *Biochem. J.*, 308: 853-858, 1995.
41. Jeanteur, P. The role of APC in colon cancer: Zeroing in on Myc. *Bulletin du Cancer (France)*, 85: 925-928, 1998.
- 10 42. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc. Natl. Acad. Sci. U.S.A.*, 92: 3046-3050, 1995.
43. Klemsz, M., Hromas, R., Raskind, W., Bruno, E., and Hoffman, R. PE-1, a novel ETS oncogene family member, localizes to chromosome 1q21-q23. *Genomics*, 20: 291-294, 1994.
- 15 44. Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J.G., Bustelo, X.R., and Freire, M. The expression of prothymosin alpha gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. *J. Biol. Chem.*, 264: 8451-8454, 1989.
45. Bories, D., Raynal, M.C., Solomon, D.H., Darzynkiewicz, Z., and Cayre, Y.E. Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukemia cells. *Cell*, 59: 959-968, 1989.
- 20 46. Kao, C.T., Lin, M., O'Shea-Greenfield, A., Weinstein, J., and Sakamoto, K.M. Over-expression of p55Cdc inhibits granulocyte differentiation and accelerates apoptosis in myeloid cells. *Oncogene*, 13: 1221-1229, 1996.
47. Landberg, G. and Roos, G. The Cell Cycle in Breast Cancer. *APMIS*, 105: 575-589, 1997.
- 25 48. Perlaky, L., Valdez, B.C., Busch, R.K., Larson, R.G., Jhiang, S.M., Zhang, W.W., Brattain, M., and Busch, H. Increased growth of NIH/3T3 cells by transfection with human p120 complementary DNA and inhibition by a p120 antisense construct. *Cancer Res.*, 52: 428-436, 1992.
- 30 49. Zhuang, S.H. and Burnstein, K.L. Antiproliferative Effect of 1alpha,25-dihydroxyvitamin D3 in Human Prostate Cancer Cell Line LNCaP Involves Reduction of Cyclin-dependent Kinase 2 Activity and Persistent G1 Accumulation. *Endocrinology*, 139: 1197-1207, 1998.
50. Chan, A.M., Takai, S., Yamada, K., and Miki, T. Isolation of a novel oncogene, NET1, from neuroepithelioma cells by expression cDNA cloning. *Oncogene*, 12: 1259-1266, 1996.
- 35 51. Ahn, N.G. and Tolwinski, N.S. U0126: An Inhibitor of MKK/ERK Signal Transduction in Mammalian Cells. *Promega Notes*, 71: 4-13, 1999.

65. Gu, Z., Moerschell, R.P., Sherman, F., and Goldfarb, D.S. NIP1, a gene required for nuclear transport in yeast. *Proc. Natl. Acad. Sci. U.S.A.*, 89: 10355-10359, 1992.
- 5 66. Phan, L., Zhang, X., Asano, K., Anderson, J., Vornlocher, H.P., Greenberg, J.R., Qin, J., and Hinnebusch, A.G. Identification of a translation initiation factor 3 (eIF3) core complex, conserved in yeast and mammals, that interacts with eIF5. *Mol. Cell Biol.*, 18: 4935-4946, 1998.
67. Kleeff, J., Ishiwata, T., Friess, H., Buchler, M.W., Israel, M.A., and Korc, M. The helix-loop-helix protein Id2 is overexpressed in human pancreatic cancer. *Cancer Res.*, 58: 3769-3772, 1998.
- 10 68. Mielnicki, L.M., Hughes, R.G., Chevray, P.M., and Pruitt, S.C. Mutated Atf4 suppresses c-Ha-ras oncogene transcript levels and cellular transformation in NIH3T3 fibroblasts. *Biochem. Biophys. Res. Commun.*, 228: 586-595, 1996.
- 15 69. Shimizu, N., Ohta, M., Fujiwara, C., Sagara, J., Mochizuki, N., Oda, T., and Utiyama, H. A gene coding for a zinc finger protein is induced during 12-O-tetradecanoylphorbol-13-acetate-stimulated HL-60 cell differentiation. *J. Biochem. (Tokyo.)*, 111: 272-277, 1992.
- 20 70. Shimizu, N., Ohta, M., Fujiwara, C., Sagara, J., Mochizuki, N., Oda, T., and Utiyama, H. Expression of a novel immediate early gene during 12-O-tetradecanoylphorbol-13-acetate-induced macrophagic differentiation of HL-60 cells. *J. Biol. Chem.*, 266: 12157-12161, 1991.
71. Dolcetti, R., Zancai, P., Cariati, R., and Boiocchi, M. In vitro effects of retinoids on the proliferation and differentiation features of Epstein-Barr virus-immortalized B lymphocytes. *Leuk. Lymphoma*, 29: 269-281, 1998.
- 25 72. Tsuzuki, T., Izumoto, S., Ohnishi, T., Hiraga, S., Arita, N., and Hayakawa, T. Neural cell adhesion molecule L1 in gliomas: correlation with TGF-beta and p53. *J. Clin. Pathol.*, 51: 13-17, 1998.
73. Young, C.S., Kitamura, M., Hardy, S., and Kitajewski, J. Wnt-1 induces growth, cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. *Mol. Cell Biol.*, 18: 2474-2485, 1998.
- 30 74. Cai, T.Q. and Wright, S.D. Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. *J. Exp. Med.*, 184: 1213-1223, 1996.
- 35 75. De Deyne, P.G., O'Neill, A., Resneck, W.G., Dmytrenko, G.M., Pumpkin, D.W., and Bloch, R.J. The Vitronectin Receptor Associates with Clathrin-coated Membrane Domains Via the Cytoplasmic Domain of its beta5 Subunit. *J. Cell Sci.*, 111: 2729-2740, 1998.
76. Milner, R. and Ffrench-Constant, C. A developmental analysis of oligodendroglial integrins in primary cells: changes in alpha v-associated beta subunits during differentiation. *Development*, 120: 3497-3506, 1994.

B1, Ki-67, proliferating cell nuclear antigen, and p34(cdc2). Cancer, 85: 1569-1576, 1999.

The contents of the publications and patents referenced herein are incorporated herein by reference in their entirety.

5



9. A method according to claim 7, wherein said cancer cells are glioblastomas.
10. A method according to claim 7, wherein said cancer cells are human brain tumor cells.
11. A method according to claim 7, wherein said cancer cells are human astrocytoma cells.
12. A method according to claim 7, wherein said cancer cells are human colonic carcinoma cells.
13. A method according to claim 7, wherein said cancer cells are human colonic adenocarcinoma cells.
14. A method according to claim 7, wherein said cancer cells are human ovarian carcinoma cells.
15. A method according to claim 7, wherein said cancer cells are human hepatocellular carcinoma cells.
16. A method of decreasing the expression of peripheral-type benzodiazepine receptor mRNA in cancer cells in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.
17. A method of increasing the expression of c-Myc protooncogene in a patient in need of such increasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.
18. A method of decreasing the expression of cell cycle regulators prothymosin- $\alpha$ , CDK2, p53, myeloblastin and p120 proliferating-cell nuclear antigen in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.
19. A method of decreasing the expression of intracellular signal transduction modulators NET1 and ERK2, in a patient in need of such decreasing, which comprises administering an effective amount of Ginkgo biloba extracts or isolated Ginkgolide B to said patient.
20. A method of decreasing the expression of apoptosis-related products Adenosine A2A Receptor, Flt3 ligand, Grb2, Clusterin, RXR- $\beta$ , Glutathione S-transferase P, N-Myc, TRADD, SGP-2 and NIP-1, in a patient in need of such decreasing, which comprises

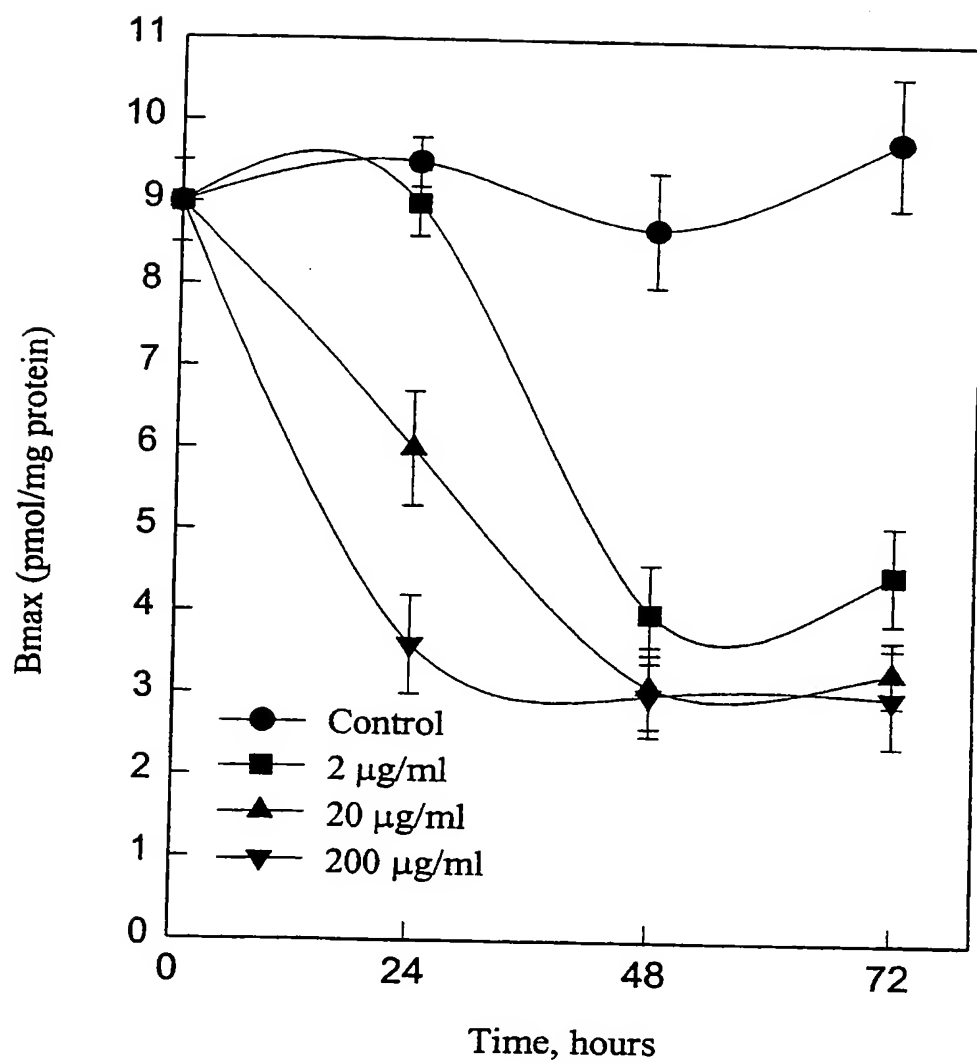


Figure 1

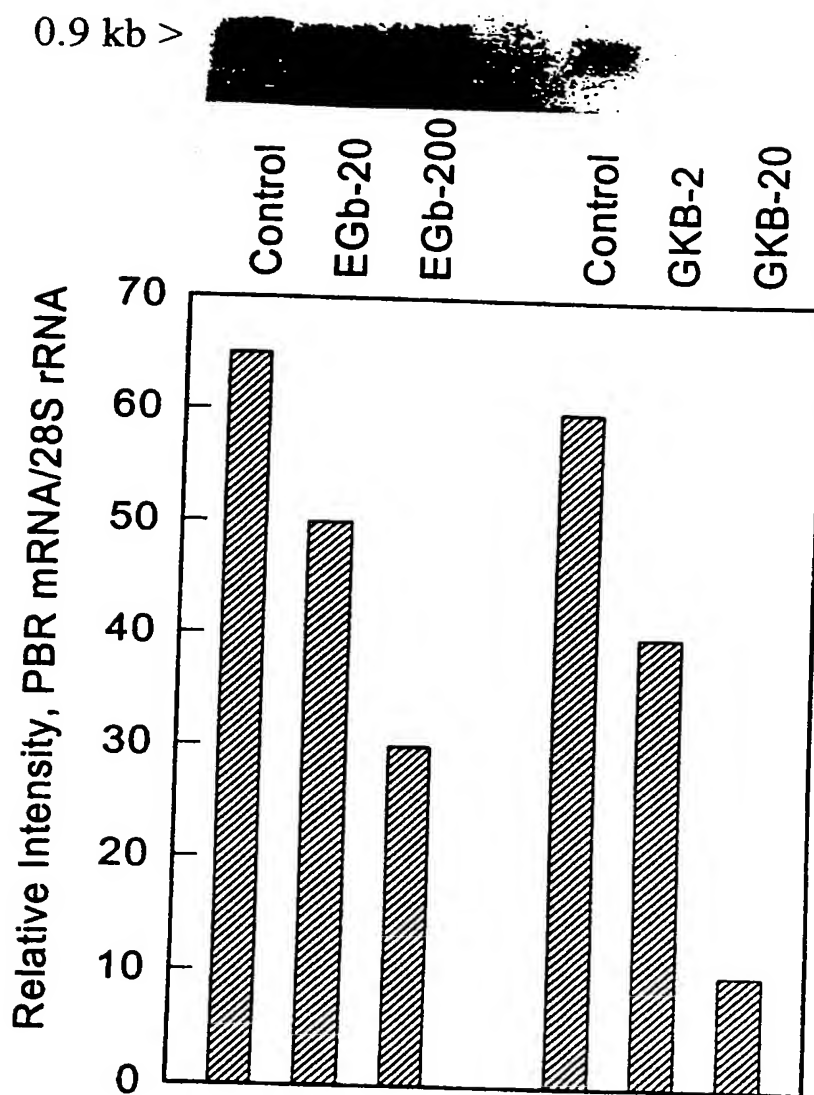


Figure 3

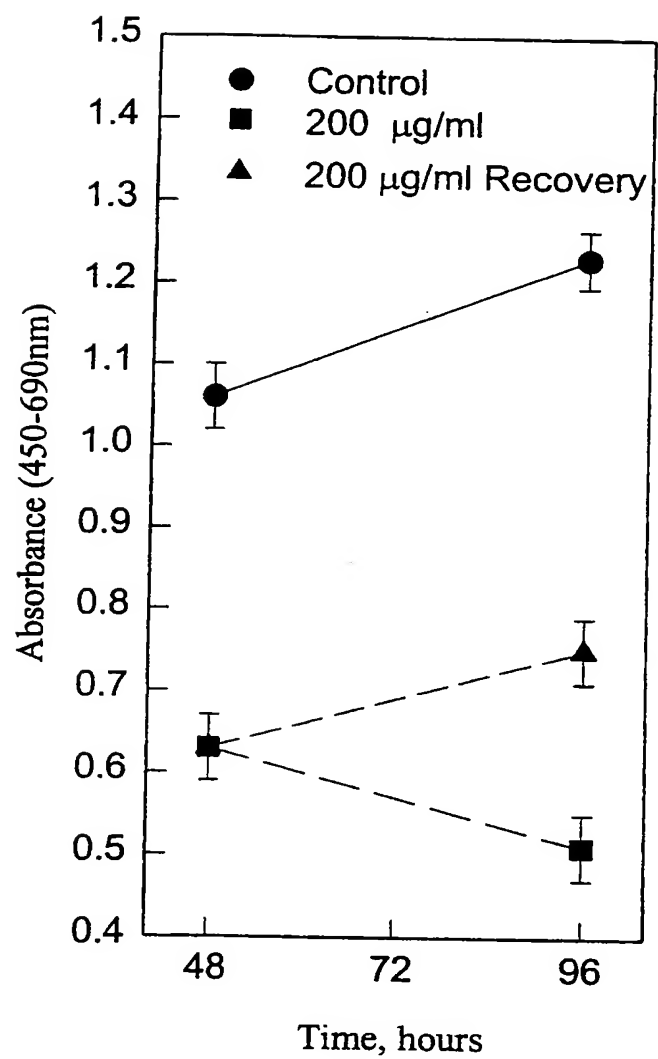


Figure 5: Right

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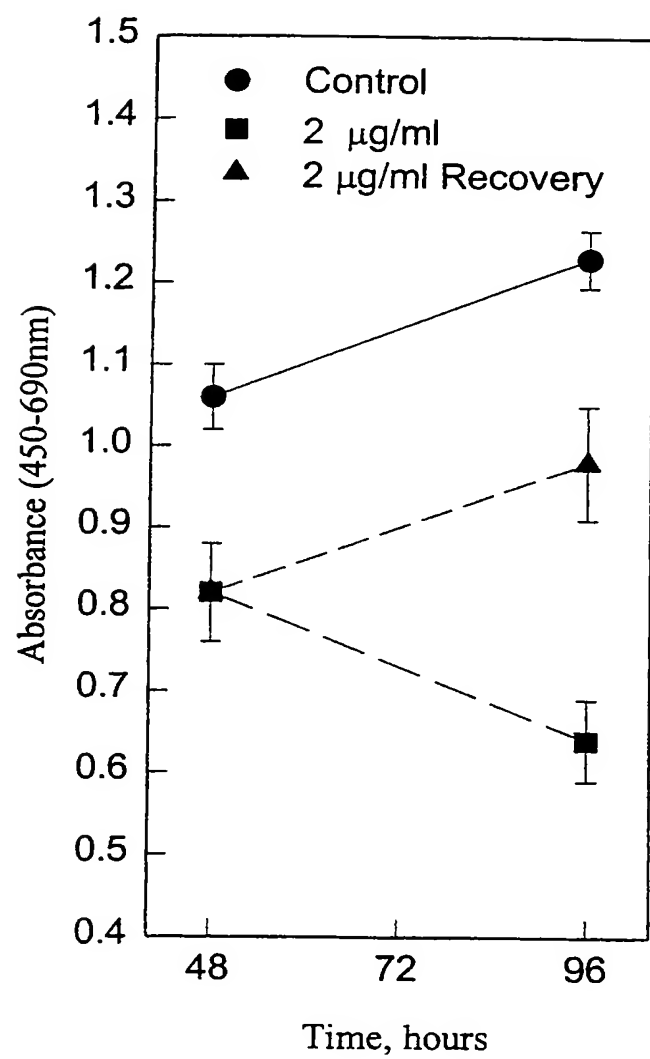


Figure 5: Left

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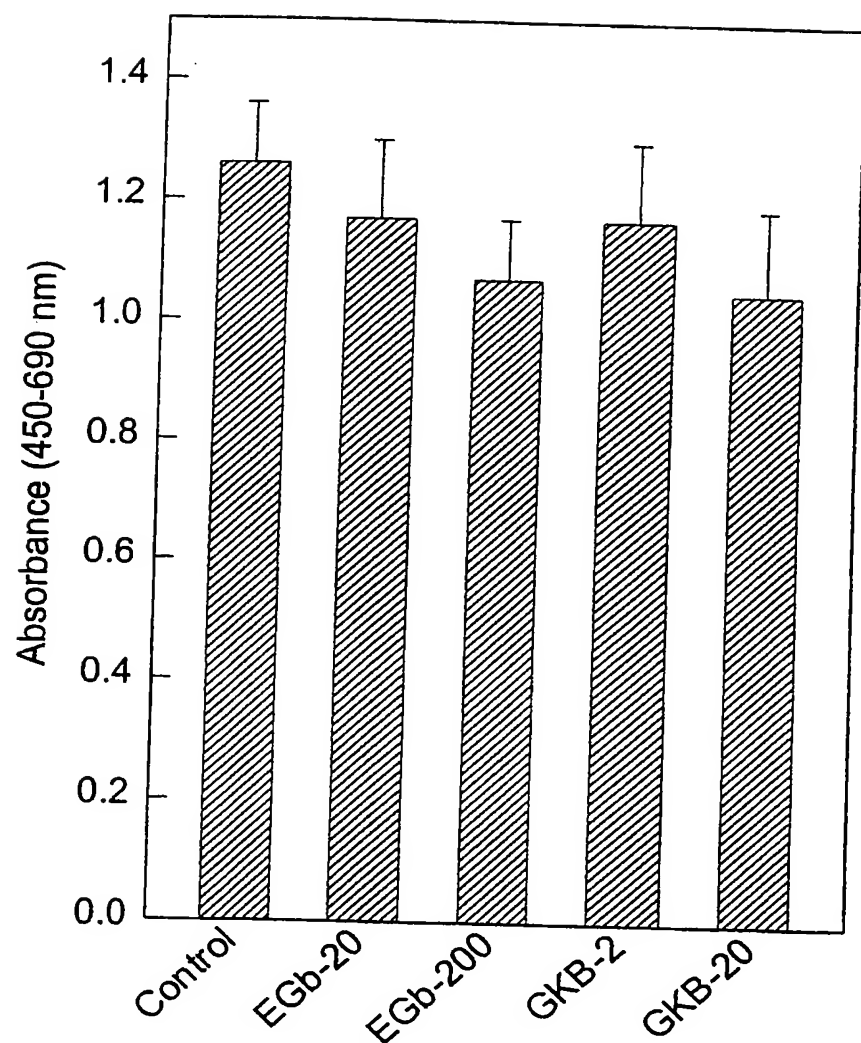


Figure 7

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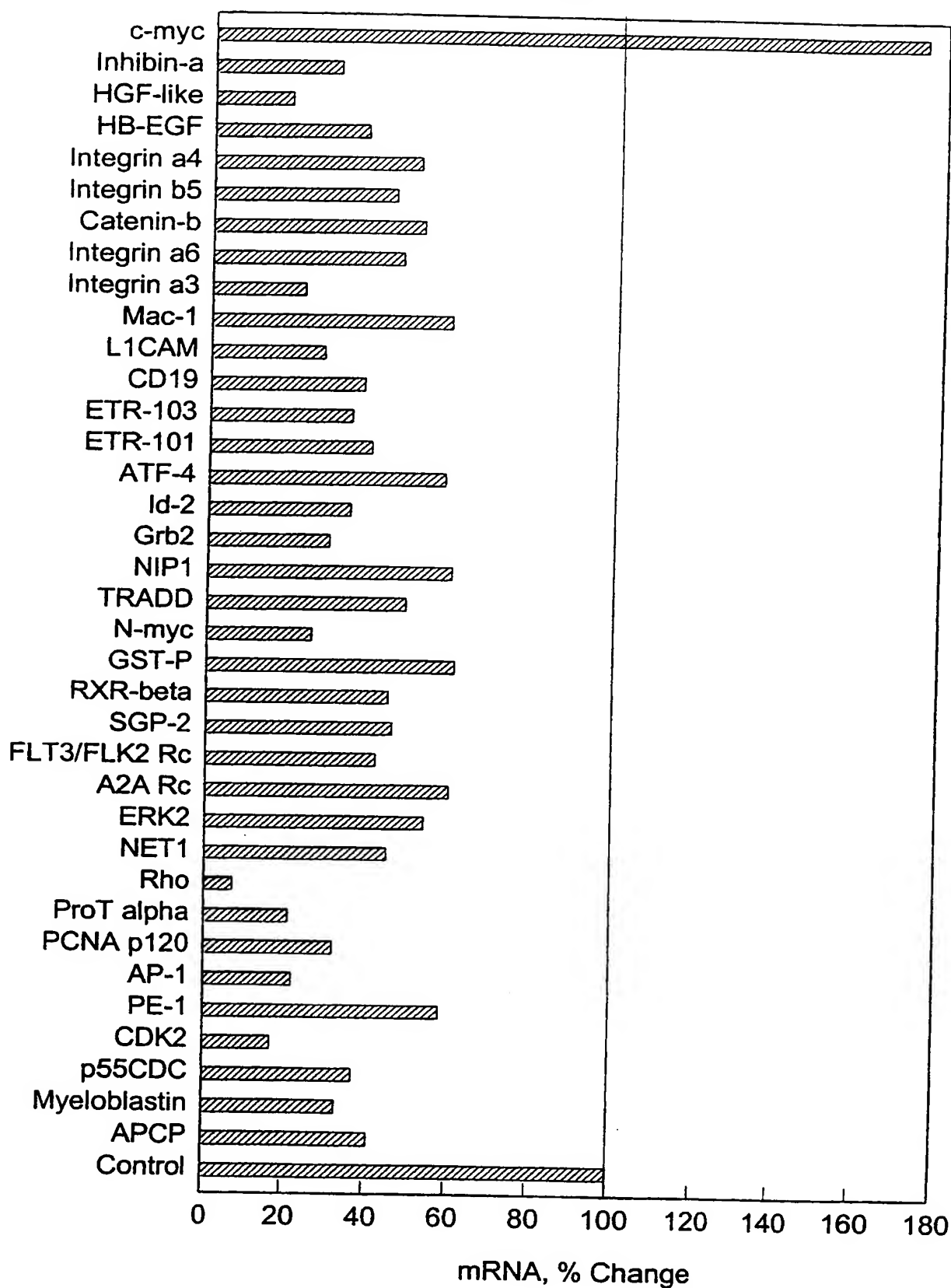


Figure 9

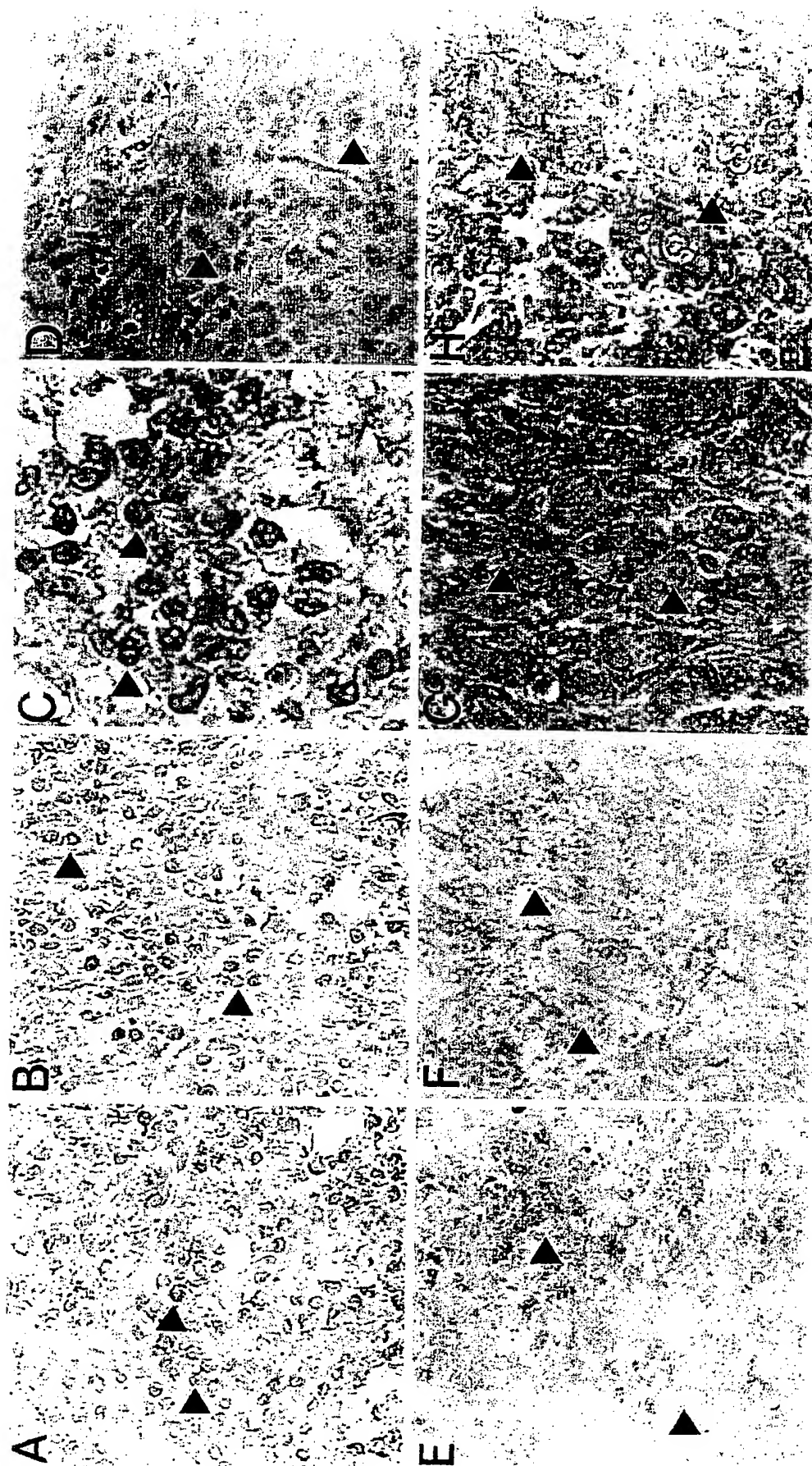


Figure 11



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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Continuation of Box I.1

Claims Nos.: 1-24

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy